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A Complex Response Element in Intron 1 of the Androgen-regulated 20-kDa Protein Gene Displays Cell Type-dependent Androgen Receptor Specificity*

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The androgen-regulated 20-kDa protein gene consists of four exons that code for a major secretory protein of rat ventral prostate. Analysis of its potential cis-acting transcriptional regulatory elements revealed that a large intron 1 region (In-1) had stronger androgen response element (ARE) activity than did the 5′-flanking DNA. In cotransfected CV1 cells, In-1 and its most active subfragment In-1c functioned as AREs but not glucocorticoid response elements (GRE). Nevertheless several ARE/GRE-like partial palindromic sequences are present in In-1c, and it bound both androgen receptors and glucocorticoid receptors in mobility shift assays. A cluster of three ARE/GRE-like sequences contained within a 39-base pair sequence of In-1c had both ARE and GRE activities when analyzed as an isolated oligonucleotide, suggesting that other elements within In-1c determined its ARE specificity. In addition to ARE/GRE-like sequences, In-1c contains putative response elements for the transcription factors AP1, CREB, AP2, OCT-1, C/EBP, and a number of inverted and direct repeats. The ARE specificity of In-1c observed in CV1 cells was diminished in PC3 and HeLa cells transiently cotransfected with an androgen receptor or glucocorticoid receptor expression vector together with an In-1c reporter vector; however, the ARE activity of In-1c was greater than its GRE activity in these cell lines. Interestingly, a 131-base pair subfragment of In-1c retained ARE specificity in all three cell lines.

Regulatory effects of steroid hormone receptors on gene transcription are initiated by their interactions with nucleotide sequences referred to as hormone response elements (1–3). These elements are recognized by the receptor DNA binding domain, a cysteine-rich region containing two zinc fingers (4). The subfamily of nuclear receptors, which includes androgen (AR),[1] glucocorticoid (GR), progesterone, and mineralocorticoid receptors, contains a high degree of sequence similarity within the DNA binding domains of the receptors (5). This sequence homology is reflected in their common interactions with a similar class of response elements consisting of 15 bp partial palindromic sequences (3, 6–12). Studies on the androgen-regulated prostatein C3 subunit gene identified a potent androgen response element (ARE) AGTACGn$_3$TGTTCT (9, 10, 13) within the first intron which also functions as a glucocorticoid response element (GRE) in transient cotransfection assays (14). Several weaker elements were identified within the C3 first intron and 5′-flanking regions, and these too displayed both ARE and GRE activities (10, 14). This set of simple response elements provides a plausible basis for the overlapping androgen and glucocorticoid effects on gene expression which have been demonstrated in rat ventral prostate. However, the predominant androgen dependence of gene expression in rat prostate suggests that there exist more specific AREs that respond selectively to androgen.

Thus far an AR-specific single 15-bp palindromic sequence has not been identified, but receptor specificity may be determined by more complex response elements. Recent studies on the AR, GR, progesterone receptor subfamily have shown that transactivation can be mediated by complex elements that include multiple simple steroid response elements (15-bp partial palindromes or half-sites) together with recognition sequences for other transcription factors. A response element of this type could provide a framework for modulation of receptor transactivation by other factors through both protein-protein and protein-DNA interactions (3, 12, 15–21).

Herein we report on a complex element that responds selectively to the androgen receptor. The complex androgen response element is located in the first intron of the androgen-regulated 20-kDa protein gene that codes for one of the major secretory proteins of rat ventral prostate and lacrimal gland (22–24). This intron element conferred androgen but not glucocorticoid receptor responsiveness to a heterologous promoter in transient cotransfection experiments utilizing monkey kidney CV1 cells. In the human prostate cancer cell line PC3 and in HeLa cells, both AR and GR transactivation was

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L12454.

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[1] The abbreviations used are: AR, androgen receptor; GR, glucocorticoid receptor; bp, base pair(s); ARE, androgen response element; GRE, glucocorticoid response element; PCR, polymerase chain reaction; PIPES, 1,4-piperazinediethanesulfonic acid; CAT, chloramphenicol acetyltransferase; DBD, DNA binding domain; C/EBP, CCAAT and enhancer core binding protein; CREB, cyclic-adenosine monophosphate response element binding protein.

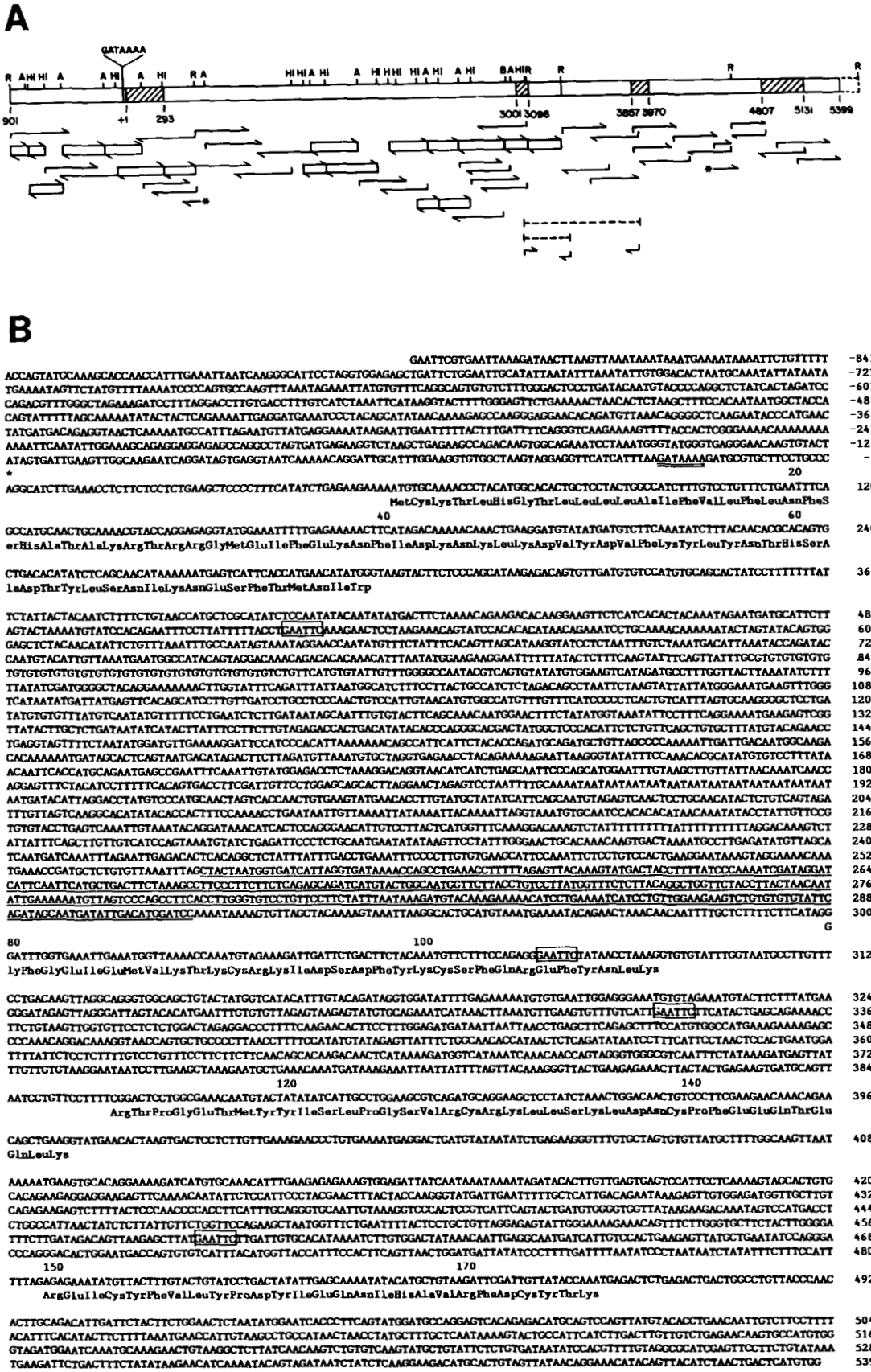

FIG. 1. **Nucleotide sequence of the rat 20-kDa protein gene.** *Panel A*, schematic diagram of the 20-kDa protein gene and its sequencing strategy. The sequence GATAAAA is the putative TATA box. *Cross-hatched bars* are exons. *Open bars* are 5′-flanking, introns 1, 2, 3, and 3′-flanking regions. Residue numbers are indicated relative to the predicted mRNA start site. The restriction enzyme sites shown were used in sequencing: *R*, *Eco*RI; *Hi*, *Hin*fI; *A*, *Alu*I; *H*, *Hinc*II. The sequence between nucleotides 3001 and 5399 was analyzed in

FIG. 2. **Primer extension analysis of the transcription start site of the 20-kDa protein gene.** *Lanes 1–4*, sequencing ladders of M13mp19 using the 17-mer sequencing primer −20 in the order of G, A, C, T. *Lanes 5–7*, the products of extension using 1.0, 0.1, and 0.01 μg of rat ventral prostate poly(A) RNA as template. The *arrow* indicates the position of the longest DNA product which corresponds to a G residue of M13mp19, 372 nucleotides from the 5′ start point of the 17-mer sequencing primer −20. This located the mRNA transcription start site at an A residue of the genomic DNA sequence shown in Fig. 1.

observed; however, the response to AR remained greater than GR.

MATERIALS AND METHODS

Genomic Clones and DNA Sequencing—*Eco*RI and *Hae*III partially digested rat genomic DNA libraries constructed in λ Charon 4A were provided by T. Sargent, R. Wallace, and J. Bonner (California Institute of Technology). Libraries were screened for the 20-kDa gene using two *Eco*RI cDNA fragments (nucleotides 237–352 and 353–809) as probes (22, 25). The DNA fragments were labeled with [α-^{32}P] dCTP by the multiprime DNA labeling system (Amersham Corp.). Genomic clones were digested with *Eco*RI and subjected to Southern blot analysis using the cDNA probes described above.

*Eco*RI genomic DNA fragments hybridizing to cDNA probes were cloned into M13mp19 or pGEM3zf(+) at the *Eco*RI site. Subfragments generated by restriction with different enzymes were blunt ended using T4 DNA polymerase and cloned into M13mp19 or pGEM3zf(+) at the *Sma*I site. Sequencing in M13mp19 was performed by the standard dideoxy chain termination method (26). Sequencing in pGEM3zf(+) was performed using a series of synthetic oligonucleotide primers and Sequenase according to the manufacturer's directions (U. S. Biochemical Corp.).

Double-stranded DNA was synthesized by polymerase chain reaction (PCR), purified by agarose gel electrophoresis, and cloned into pGEM3zf(+) for sequencing. Single-stranded DNA was synthesized by asymmetric PCR, purified by centrifugation in Centricon-30 or -100 (Amicon Division, W. R. Grace & Co.), and sequenced using Sequenase.

Primer Extension Analysis—The 5′ start site of mRNA transcription was determined by primer extension analysis as described previously (27). Total RNA was isolated from Sprague-Dawley rat (250–350 g) ventral prostate using the acid guanidinium thiocyanate-phenol-chloroform method (28) and enriched for poly(A) RNA by oligo(dT)-cellulose chromatography (25). The 5′ end ^{32}P-labeled oligonucleotide, 5′-CCCTCTGGAAAGAACATTTGTAGAAGTCAGAACAATCTTTCTACATTTGGTTTTAACC-3′ (nucleotides 3079–3021 in Fig. 1*B*) was incubated with decreasing amounts (1.0–0.01 μg) of rat ventral prostate poly(A) RNA in 50% formamide, 0.4 M NaCl, 1 mM EDTA, and 40 mM PIPES, pH 6.4, at 85 °C for 10 min and placed at 42.5 °C for 6 h. After ethanol precipitation, the oligonucleotide was extended with avian myeloblastosis virus reverse transcriptase (Promega) on the 20-kDa mRNA. DNA products were subjected to electrophoresis on a 5% sequencing gel in parallel with dideoxy sequencing ladders of M13mp19 size markers.

Plasmid Construction for Functional Analysis—DNA fragments or synthetic oligonucleotides were cloned into polylinker regions of the reporter vector ptkCAT 5′ of the thymidine kinase promoter and chloramphenicol acetyltransferase (CAT) gene or the reporter vector pCAT 5′ of the CAT gene. ptkCAT and pCAT were derived from $pBLCAT_2$ and $pBLCAT_3$ respectively, by removal of the 217-bp *Nde*I-*Hin*dIII fragment containing a potential GRE sequence (29). All constructs were verified by double-stranded sequencing using oligonucleotide primers. Clone pFL-1 contains the flanking region fragment FL-1, nucleotides −895 to −38. FL-1 was prepared using PCR primers 5′-GTAATTAAAGATAACTTAAG-3′ (nucleotides −895 to −875) and 5′-CCTCCTACTTAGCCAGACCTTCC-3′ (nucleotides −38 to −60) on the 1,426-bp *Eco*RI fragment. Fragment FL-1 was cloned into the T4 DNA polymerase-blunted *Bam*HI site of ptkCAT. The flanking region containing nucleotides −901 to +8 was generated by cleaving the 1,426-bp *Eco*RI fragment with *Mbo*II and cloned into the *Bam*HI site of pCAT by blunt end ligation. Clone pln-1 containing the intron 1 fragment In-1 was generated by restriction of the 2,559-bp *Eco*RI fragment with *Bam*HI and cloning into the *Bam*HI site of ptkCAT by blunt end ligation.

Clone pIn-1a contains the intron 1 subfragment In-1a (nucleotides 1419–1779). The fragment was generated by restriction digestion of the fragment In-1 with *Hinc*II then *Alu*I. In-1a was cloned into the *Bam*HI site of ptkCAT by blunt end ligation. Clones pIn-1b and pIn-1c were constructed similarly but contain the intron 1 subfragments In-1b, nucleotides 2013–2290, and In-1c, nucleotides 2549–2907, respectively. However, pIn-1c was first cloned in reverse orientation, cut with *Bam*HI, and reinserted in the correct orientation to preserve the *Bam*HI sites. Clone pD1 contains the larger subfragment (D1) of In-1c (nucleotides 2680–2907) cleaved by *Dde*I and cloned into the *Sal*I site of ptkCAT by blunt end ligation. Clone pD2 contains the smaller subfragment (D2) of In-1c, nucleotides 2549–2680, obtained by *Dde*I cleavage. Clone pN39 contains a 39-bp oligonucleotide of fragment In-1c, nucleotides 2684–2722. The oligonucleotide was prepared with *Sal*I sites at both ends and cloned into *Sal*I site of ptkCAT. pCMVrAR is a pCMV1 vector containing full-length rat AR cDNA (10, 30). pCMVrGR was constructed by cloning the full-length rat GR (31) into pCMV1.

Cell Culture, Transfection, and CAT Assay—African green monkey kidney cells, CV1, were maintained at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium-H supplemented with 5% fetal bovine serum. Human prostate cancer cells, PC3, were maintained at

pGEM3Zf(+) using synthetic oligonucleotide primers. Each sequence was determined on two different clones. The *arrows* indicate the sequencing directions. Sequences verifying the continuity of adjacent *Eco*RI fragments are indicated by *arrows* with *asterisk* markers and were obtained by the Sequenase method using ^{32}P-labeled oligonucleotide primers and genomic DNA in λ phage as template. The *broken-lined* regions indicate the PCR DNA products from rat genomic DNA using primers 5′-CTGACTTCTACAAATGTTCTTTCCAGAGGG-3′ (nucleotides 3050–3079) and 5′-CCTCTAGTCCAGAGAGGAACACCAACTTAC-3′ (nucleotides 3395–3366) or 5′-AGATAGGAGCTTCCT GCATCTGACGCTTCC-3′ (nucleotides 3925–3896). Both fragments contain the third *Eco*RI fragment (1,255 bp) which is in clone H8 (See "Results"). Sequences of the end portions of the PCR DNA were determined directly from asymmetric PCR products and by sequencing the smaller double-stranded PCR DNA fragment cloned in pGEM3zf(+). *Panel B*, nucleotide sequence of the 20-kDa protein gene in *panel A*. The mRNA start site is marked by * and numbered as +1. The putative TATA box is *double-underlined* and located centrally at −22. Coding regions are displayed with deduced amino acid sequences. The first intron region In-1c containing the complex ARE is *underlined*. *Eco*RI restriction sites are *boxed*.

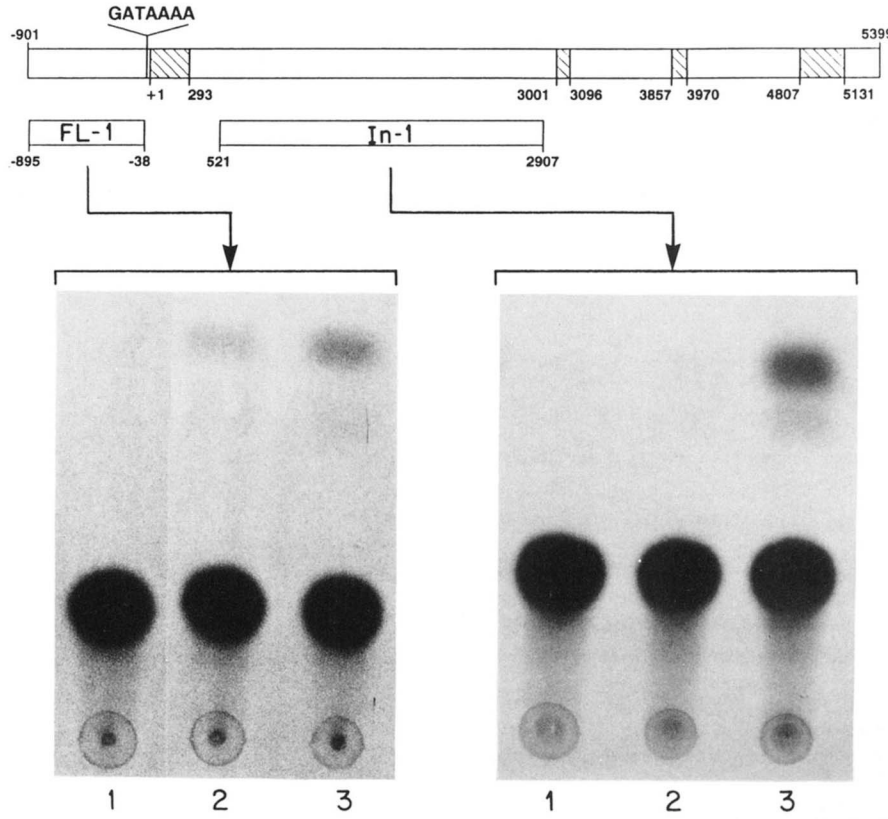

FIG. 3. **Androgen-dependent enhancer activities of the 5′-flanking region and intron 1 fragment In-1 in CV1 cells.** Schematic diagram of the 20-kDa protein gene and the two fragments, FL-1 and In-1, cloned into the reporter vector ptkCAT for assay of androgen-dependent enhancer activity (see "Materials and Methods" for construction). CV1 cells were cotransfected with the androgen receptor expression vector pCMVrAR (1 μg) and (5 μg) of pFL-tkCAT containing the flanking region FL-1 or pIn-1-tkCAT containing the intron 1 fragment In-1. *Lane 1*, recombinant reporter vector in the presence of 50 nM R1881. *Lane 2*, recombinant reporter vector plus pCMVrAR without R1881. *Lane 3*, the same as *lane 2* but with 50 nM R1881. CAT activities were measured in CV1 cell extracts as described under "Materials and Methods."

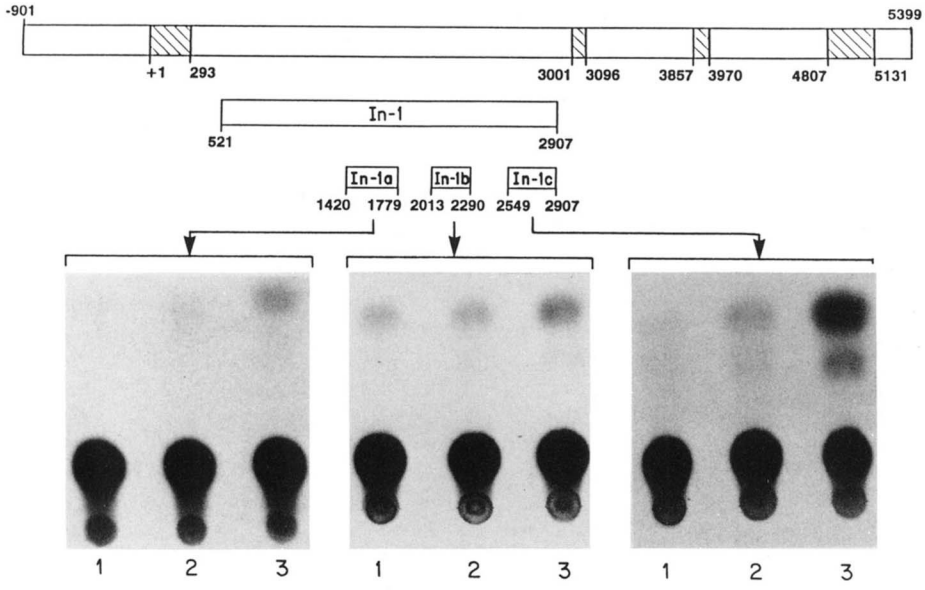

FIG. 4. **Androgen-dependent enhancer activities of intron 1 subfragments in CV1 cells.** The diagram at the *top* indicates relative locations of In-1 subfragments In-1a, In-1b, In-1c, and *arrows* point to CAT assays with these fragments shown below. CV1 cells were cotransfected with 5 μg of recombinant reporter vector (tkCAT) containing In-1a, In-1b, or In-1c, and 1 μg of the androgen receptor expression vector, pCMVrAR. *Lane 1*, recombinant reporter vector (5 μg) in the presence of 50 nM R1881. *Lane 2*, recombinant reporter vector plus pCMVrAR (1 μg) without R1881. *Lane 3*, the same as *lane 2* but with 50 nM R1881. Data shown are representative of at least five experiments. CAT activities were measured in CV1 cell extracts as described under "Materials and Methods."

37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium and F-12 medium supplemented with 5% fetal bovine serum. Human cervical carcinoma cells, HeLa, were maintained in Eagle's minimal essential medium supplemented with 5% fetal bovine serum. One day before transfecting, cells were plated in 10-cm culture dishes at 1.2×10^6 cells/dish and grown in the same medium for 20 h when they were 70–80% confluent. (See the legend to Table III for modifications of the protocol in experiments comparing regulation in CV1, PC3, and HeLa cells.) Cells were transfected with 1 μg of receptor expression vector DNA, pCMVrAR or pCMVrGR and 5 μg of reporter plasmid DNA using the $CaPO_4$ method as described previously (10). Cells were washed twice, placed in Dulbecco's modified Eagle's medium-H without phenol red and supplemented with 0.2% fetal bovine serum in the presence or absence of the indicated concentration of synthetic androgen, R1881 or synthetic glucocorticoid, dexamethasone. After incubation for 20 h, the medium was replaced with or without hormone at the same concentration. Cells were incubated for another 24 h, harvested in phosphate-buffered saline (0.15 M NaCl, 0.01 M Na_2HPO_4, pH 7.2), and assayed for CAT activity as described by Gorman *et al.* (32) except that the cells were broken by freeze-thaw three times. Thin layer plates were exposed on Kodak X-Omat AR film for 24–72 h. For quantitation, the radioactive spots were cut out from the plates and counted in a liquid scintillation counter. Stimulation of CAT activity was expressed as fold increase over background and was based on at least five independent experiments.

Mobility Shift DNA Binding Assay—DNA receptor binding was analyzed by gel mobility shift using AR or GR DNA binding domain polypeptides and was performed as described previously (10). Specific binding of receptor to DNA was demonstrated using the protein A-purified IgG fraction of anti-AR antiserum (AR-52) or unpurified anti-GR antiserum (BuGR-2). 20-kDa protein gene fragments used in the mobility shift assay were inserts of ptkCAT vectors removed

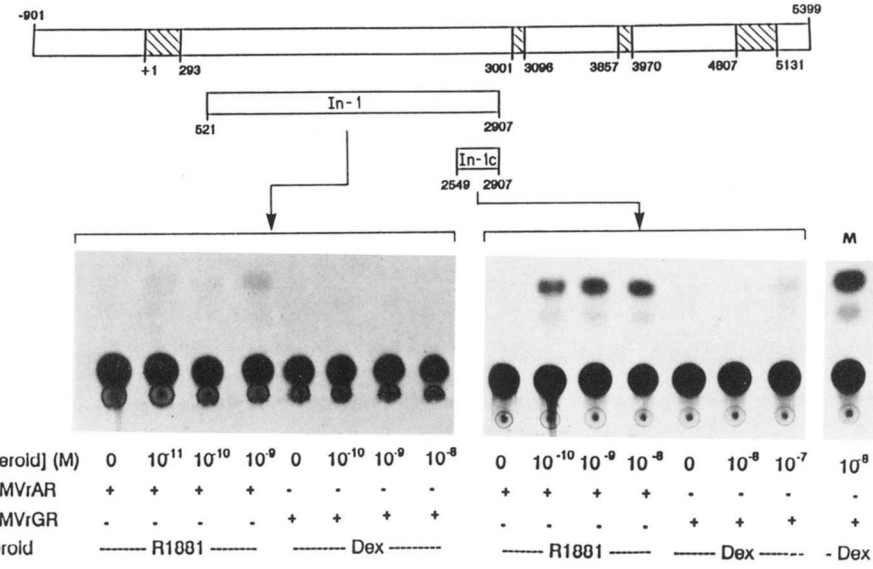

FIG. 5. **Comparison of androgen- and glucocorticoid-dependent enhancer activities of intron 1 fragment In-1 and subfragment In-1c in CV1 cells.** The diagram at the *top* indicates In-1 and its subfragment In-1c with *arrows* pointing to their respective CAT assays below. CV1 cells were cotransfected with pIn-1-tkCAT or pIn-1c-tkCAT (5 μg each) and pCMVrAR or pCMVrGR (1 μg each) in the presence or absence of increasing concentrations of R1881 or dexamethasone. *Lane M*, reporter vector containing the 5′ most GRE of the mouse mammary tumor virus long terminal repeat as a positive control.

by restriction enzyme digestion to generate 5′-protruding ends and purified by electroelution from polyacrylamide gels. The 5′-protruding ends were filled in with [^{32}P]dCTP (Amersham Corp.) using Klenow fragment of DNA polymerase I (Life Technologies, Inc.).

Overexpression of Recombinant Androgen and Glucocorticoid Receptor—The rat GR DNA binding domain (DBD) (amino acid codons 407–556) was excised from the expression vector pT7X556 by *Nde*I and *Cla*I digestion and subcloned into the T7 RNA polymerase-dependent expression vector pET16b (Novagen) at the same restriction sites. In addition to non-GR residues present in T7X556, the resulting construct, pET16b-rGR-DBD, contained 19 non-GR amino acids at the amino terminus contributed by pET16b including 10 histidine residues which permit rapid purification using a nickel-containing affinity resin.

A 510-bp DNA fragment corresponding to rat AR amino acid codons 495–665 encompassing the DNA binding domain and hinge region of AR was generated by PCR amplification using oligonucleotides that introduce *Bam*HI sites at either end of the amplified fragment. The PCR product was digested with *Bam*HI and inserted into the expression vector pET16b. The resulting expression vector, pET16b-rAR-DBD, contained 19 non-rAR amino acids at the amino terminus including 10 histidine residues and 19 non-AR amino-acids at the carboxyl terminus including a stop codon.

BL21(DE3)pLysS, a strain carrying a stable integrant of T7 RNA polymerase under the control of the *lac* UV5 promoter, was transformed with pET16b-rGR-DBD or pET16b-rAR-DBD, grown at 30 °C in LB medium containing carbenicillin and chloramphenicol, and induced at midlogarithmic growth (A_{600} = 0.6–0.8) by the addition of isopropyl-1-thio-β-D-galactopyranoside to 1 mM. After 2 h of induction, cells were collected by centrifugation, resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 10% glycerol, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 1 μg/ml aprotinin), and 5 mM imidazole, 0.04 ml/ml of culture) and sonicated on ice for three 10-s intervals. After 15 min on ice, sodium deoxycholate was added to 0.05% and the suspension stirred for 30 min in an ice bath. The suspensions were centrifuged at 40,000 × *g* for 30 min at 4 °C. Supernatants were filtered through a 0.45-μm filter and mixed with a nickel-containing affinity resin (His-Bind™, Novagen), 1 ml of resin/10 ml of supernatant, for 1 h at 4 °C by inversion. Resin suspensions were packed into columns, washed sequentially with 10 ml of lysis buffer, 10 ml of lysis buffer containing 25 mM imidazole, and 10 ml of lysis buffer containing 50 mM imidazole. Receptor proteins rGR-DBD or rAR-DBD were eluted with 5 ml of lysis buffer containing 200 mM imidazole. Protein fractions were dialyzed against $TEGDZ_{50}$ (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 50 μM $ZnSO_4$, and 50 mM NaCl) for 4 h at 4 °C, aliquoted, frozen in liquid N_2, and stored at −80 °C. Eluted protein fractions were approximately 20 and 40% rGR-DBD and rAR-DBD, respectively, as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis Coomassie Blue staining and Western blotting.

RESULTS

Structure and Nucleotide Sequence of 20-kDa Protein Gene—Two genomic clones of the 20-kDa protein gene were selected, clone R6 from the *Eco*RI partially digested library, and clone H8 from the *Hae*III partially digested library (also cloned in Charon 4A at *Eco*RI site with *Eco*RI linker). When these genomic fragments were restricted with *Eco*RI, clone R6 yielded 7 bands and clone H8 11 bands on agarose gel electrophoresis.

In Southern blot analysis, two fragments of clone R6 (2,564 and 1,423 bp, respectively) hybridized to the 5′ portion of 20-kDa protein cDNA (*Eco*RI fragment, nucleotides 237–352 in Ref. 22). The 3′ portion of the 20-kDa protein cDNA (nucleotides 353–809) hybridized with two fragments of clone H8 (1,255 bp and approximately 4 kilobases, respectively) (data not shown). Clone H8 also contained a 258-bp *Eco*RI fragment that did not hybridize with the cDNA probe. It was demonstrated by PCR that this fragment is in the second exon-intron region of 20-kDa gene (see Fig. 1*A*). The sequencing strategy and nucleotide sequence are shown in Fig. 1, *A* and *B*. Primer extension analysis (Fig. 2) together with the cDNA sequence reported earlier (22, 23) indicated that this genomic DNA sequence contains the entire 20-kDa gene sequence including 901 bp 5′ of the transcription start site with a TATA box at −22, a 293-bp first exon, 2,709-bp first intron, 96-bp second exon, 762-bp second intron, 114-bp third exon, 838-bp third intron, 325-bp fourth exon, and 267-bp nontranscribed 3′ region.

Androgen Response Element Activities of the 5′-Flanking Region and Intron 1 Fragments—Androgen-dependent enhancer activities of the 20-kDa protein gene 5′-flanking region and intron 1 fragment were compared in CV1 cells. The 5′-flanking and intron 1 fragments were cloned into pCAT and ptkCAT, respectively, both at the *Bam*HI site, and cotransfected with the rat androgen receptor expression, pCMVrAR, in the presence or absence of the synthetic androgen R1881 (50 nM). No increase in CAT activity was detected with the 5′ fragment, nucleotides −901 to +8 (created by *Mbo*II cleavage) in pCAT (data not shown). When a fragment of this region, FL-1 (nucleotides −895 to −38) without the putative TATA box, was tested in ptkCAT, CAT activity was stimulated only about 2-fold by R1881 (50 nM) (Fig. 3). A subfragment of FL-1, nucleotides −503 to −181, was also tested in ptkCAT, and CAT activity was stimulated only 2-fold by

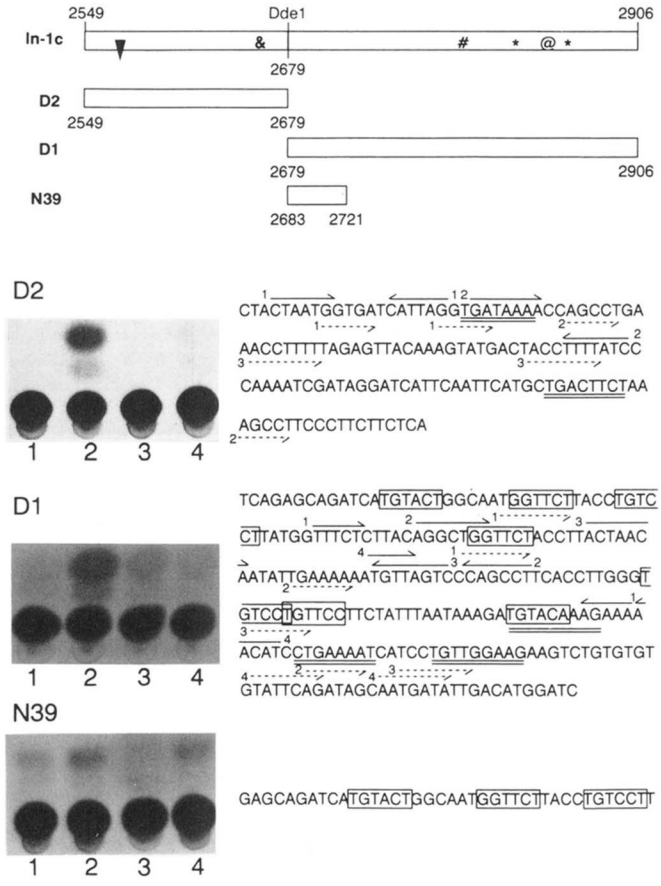

FIG. 6. **Comparison of androgen- and glucocorticoid-dependent enhancer activities of In-1c sequences D1, D2, and N39 in CV1 cells.** The diagram at the *top* shows the fragment In-1c and its subfragments D1, D2, and N39. The restriction enzyme site (*Dde*I) used to generate D1 and D2 is indicated. *Symbols* in *boxes* indicate relative positions of ARE/GREs and other transcription factor recognition sequences. (▼, AP1; &, CREB; #, AP2; *, C/EBP; @, OCT-1; also see Tables I and II). The reporter vector pD2tkCAT, pD1tkCAT, or pN39tkCAT (5 μg each) was cotransfected with expression vector pCMVrAR (*lanes 1* and *2*) or pCMVrGR (*lanes 3* and *4*) (1 μg each). CAT activity assays for each fragment are shown on the *left*. *Lanes 1* and *2* are in the absence and presence of R1881 (10 nM) respectively; *lanes 3* and *4*, in the absence and presence of dexamethasone (10 nM). On the *right* are nucleotide sequences D2, D1, and N39. ARE/GRE right half-sites are *boxed*. The consensus GRE sequence is GGTACAn_3TGTTCT (40). Transcription factor recognition sites are *double-underlined* (see also Tables I and II for ARE/GREs and transcription factor recognition sequences). *Solid overline arrows* indicate inverted repeat sequences; *broken underline arrows* indicate direct repeat sequences.

TABLE I

Potential regulatory sequences within intron 1 fragment In-1c: Nucleotide sequence of potential ARE/GREs

The 15-bp partial palindromes consist of 6-bp right and left halves separated by a 3-bp spacer. Right half-sites are underlined. The In-1c sequence is underlined in Fig. 1.

Sequence	Position[a]	Sequence	Position[a]
AGCAGATCATGTACT	2684–2698	CACCTTGGGTGTCCT	2787–2801
ACTGGCAATGGTTCT	2696–2710	TGGGTGTCCTGTTCC	2792–2806
GTTCTTACCTGTCCT	2706–2720	TAATAAAGATGTACA	2814–2828
TTACAGGCTGGTTCT	2732–2746		

[a] All sequences are located in subfragment D1 (see Fig. 6).

R1881 (data not shown). However, with the intron 1 fragment, In-1 (nucleotides 521–2907) CAT activity was stimulated 4–8-fold (Fig. 3), indicating that the first intron fragment contains a potential ARE.

TABLE II

Nucleotide sequence of potential response elements within intron 1 fragment In-1c for other transcription factors

Consensus sequences of transcription factors are from Refs. 54 and 55.

Subfragment and transcription factor	Sequence	Position	Homology with consensus
			%
D2			
AP1	TGATAAA	2570–2576	71
CREB	TGACTTCT	2655–2662	75
D1			
AP2	CCCAAGGT	2794–2787	75
C/EBP	TGTACAAAG	2823–2831	78
OCT-1	CTGAAAAT	2841–2848	75
	GACTTTTA[a]		
C/EBP	TGTTGGAAG	2854–2862	78

[a] OCT-1 consensus sequence reads 5′ to 3′ on lower strand.

The intron 1 fragment, In-1, was cleaved with the restriction enzyme *Hinc*II to yield two fragments which were digested with *Alu*I. Three fragments containing sequences resembling GREs were selected, and their androgen-dependent enhancer activities were tested (Fig. 4). Fragments In-1a, nucleotides 1420–1779, and In-1b, nucleotides 2013–2290, yielded low levels of activity (2-fold or less). However, the fragment In-1c, nucleotides 2549–2907, resulted in a 9-fold stimulation of CAT activity in initial experiments (Fig. 4). For this reason, further studies were focused on fragment In-1c.

Androgen and Glucocorticoid Response Element Activities of Intron 1 Fragment, In-1, and Subfragment, In-1c, in CV1 Cells—The ability of the intron 1 fragment, In-1, and its subfragment, In-1c, to enhance GR as well as AR transactivation was tested by cotransfection analysis in CV1 cells (Fig. 5). With the large intron 1 fragment, In-1, CAT activity increased with AR and R1881 in concentrations up to 10^{-9} M (Fig. 5). However, there was no increase in CAT activity with GR and dexamethasone up to 10^{-8} M. With In-1c there was strong androgen-stimulated CAT activity but again no response to GR and dexamethasone even at 10^{-7} M (Fig. 5). This result was in contrast to the activities of simple response elements which are capable of directing transactivation with all members of the androgen, glucocorticoid, and progesterone subfamily of nuclear receptors (3, 6–11). The sequence specific to AR was further investigated by *Dde*I restriction digestion of fragment In-1c into two fragments, D1 and D2 (Fig. 6). In addition, we synthesized a 39-bp oligonucleotide (N39) corresponding to the portion of In-1c containing a cluster of three ARE/GRE-like half-site sequences. Fig. 6 shows that the two In-1c subfragments, D1 and D2, retained androgen receptor specificity in the CAT assay (*lanes 1* and *2*) and did not mediate GR transactivation (*lanes 3* and *4*). In contrast, N39 containing three GRE-like sequences mediated increases in CAT activity in response to both R1881-AR and dexamethasone-GR (Fig. 6). The larger fragment (D1) contains 11 ARE/GRE-like sequences including the cluster of three in N39 (Fig. 6 and Tables I and II) and potential transcription factor recognition sites for AP2, C/EBP, and OCT-1. Responsiveness of N39 to both AR and GR indicates that other factors interacting with sequences outside the 39-bp region determine androgen receptor specificity in CV1 cells. The smaller fragment (D2) contains potential transcription factor recognition sites for AP1 and CREB but lacks a strong candidate ARE/GRE.

We considered the possibility of a suppressor sequence for GR or negative GRE in In-1c and tested this by insertion of

TABLE III

Effect of cell type on response element specificity of In-1c and its subfragments D1 and D2

CV1, PC3, and HeLa cells were maintained as described under "Materials and Methods" except that cells were plated in 6-cm culture dishes at 4×10^5 cells/dish. Cells were cotransfected with either the androgen or glucocorticoid receptor expression vector pCMVrAR or pCMVrGR (1 μg) together with the reporter vector pIn-1c-tkCAT, pD1tkCAT, or pD2tkCAT (5 μg) and incubated in the absence or presence of either R1881 or dexamethasone (10 nM). CAT assays were performed as described under "Materials and Methods." Numbers represent fold stimulation and are the means ± standard errors of five assays.

	CV1		PC3		HeLa	
	AR	GR	AR	GR	AR	GR
In-1c	19.8 ± 1.7	0.7 ± 0.1	10.5 ± 2.1	2.9 ± 0.8	10.7 ± 1.7	2.0 ± 0.5
D1	3.9 ± 0.7	0.5 ± 0.1	3.8 ±0.9	1.9 ± 0.5	2.4 ± 0.6	1.1 ± 0.1
D2	15.4 ± 2.5	0.5 ± 0.1	7.5 ± 0.1	0.7 ± 0.1	3.8 ± 0.5	0.6 ± 0.1

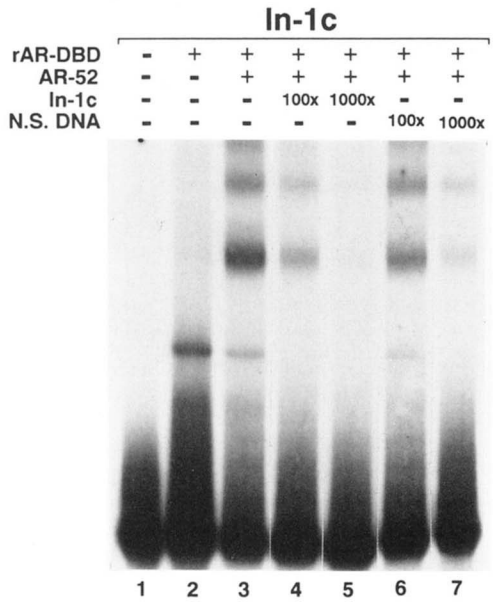

FIG. 7. **Androgen receptor binding to In-1c.** Each binding reaction contained ^{32}P end-labeled In-1c (10,000 cpm) and 1 μg of poly(dI-dC). Recombinant rAR-DBD partially purified from *Escherichia coli* was incubated with labeled probe in the presence or absence of unlabeled In-1c and loaded onto a 4% nondenaturing polyacrylamide gel. *Lane 1*, free labeled probe; *lanes 2–7*, 1.0 μg of rAR-DBD; *lanes 3–7*, 0.28 μg of antibody AR-52; *lanes 4* and *5* each contained the indicated molar excesses of unlabeled In-1c; *lanes 6* and *7* contained molar excesses of unlabeled non-specific DNA (*N.S. DNA*).

a 15-bp partial palindrome that functions as a strong ARE or GRE (oligo(C) in the C3 gene, Ref. 10). When the ARE/GRE was inserted into pIn-1ctkCAT 5′ of In-1c and cotransfected into CV1 cells with either AR or GR expression vectors, responses to dexamethasone and androgen were similar to those observed with oligo(C) alone, indicating that In-1c did not suppress the GRE activity of this ARE (data not shown). However, the strong ARE activity of oligo(C) may have overcome a suppressor of weaker hormone response elements.

Influence of Cell Type on Intron Response Element Specificity—Possible effects of cell specific factors on androgen receptor specificity of In-1c and its subfragments D1 and D2 were investigated by transient cotransfection again in CV1 cells and in two additional cell lines, PC3, a human prostate cancer cell line, and HeLa cells. None of these cell lines contained detectable levels of AR. CV1 and PC3 cells also lack GR; however, HeLa cells contain sufficient endogenous GR such that transfected mouse mammary tumor virus-CAT responded to dexamethasone without cotransfection of a GR expression vector (33). Fragment In-1c (pIn-1ctkCAT) was not as selective for androgen in PC3 and HeLa cells and mediated responses to both AR and GR; however, R1881 stimulated a greater increase in CAT activity than did dexamethasone in both cell lines (Table III). The response of subfragment D1 (pD1tkCAT) to R1881-AR was less than that of In-1c in all three cell lines. D1 was only slightly responsive to dexamethasone-GR in PC3 cells and unresponsive in CV1 and HeLa cells. On the other hand, the smaller subfragment, D2 (pD2tkCAT) retained its selectivity for androgen receptor in all three cell types, CV1, PC3 and HeLa cells. Dexamethasone-GR did not increase CAT activity with D2 in any of these cell lines and the response to R1881-AR was greater than with D1.

Androgen (AR) and Glucocorticoid (GR) Receptor Binding to Intron 1 Fragment, In-1c, and Its Subfragments, D1 AND D2—Binding of purified recombinant AR DNA binding domain polypeptide (rAR-DBD) to fragment In-1c was examined using the mobility shift DNA binding assay. When rAR-DBD was incubated with ^{32}P end-labeled In-1c (Fig. 7), a protein-DNA complex was detected which was shifted farther with anti-AR antibody (AR-52) confirming the binding of AR to this fragment. AR binding to labeled In-1c could be inhibited with unlabeled In-1c up to 1,000-fold molar excess (*lanes 4* and *5*). Nonspecific DNA was a less effective competitor of AR binding to In-1c (*lanes 6* and *7*). Recombinant GR DNA binding domain polypeptide (rGR-DBD) shifted radiolabeled In-1c as did rAR-DBD (Fig. 8*A*, *lanes 1–5*), and the anti-GR antibody (BuGR) caused a farther shift confirming the binding of GR to this element. Excess unlabeled In-1c also inhibited binding of rGR-DBD to labeled In-1c (data not shown). These results demonstrated that fragment In-1c contains recognition sequences for AR and GR. However, by comparison with the strong binding to the C3 gene first intron ARE/GRE, C3-C (10) binding of AR and GR to In-1c was weaker (Fig. 8*B*, *lanes 11–14*). We also compared rAR-DBD and rGR-DBD binding to the D1 and D2 subfragments of In-1c and the N39 sequence located at the 5′ end of D1 (Fig. 8, *A* and *B*). D1, D2, and N39 all bound both AR and GR as indicated by the shifted complexes in the presence of receptor antibody. However, using amounts of protein and antibody that yielded similar AR and GR binding to C3-C, binding of GR to In-1c and its subfragments was less than that of AR, especially with D2 and N39 (Fig. 8, *A* and *B*).

DISCUSSION

The 20-kDa protein gene was cloned from a rat genomic DNA library and found by sequence analysis to contain four exons, a large intron 1 and smaller introns 2 and 3. Within intron 1 is a region containing multiple partial palindromic GRE-like sequences, suggesting that it might function as a

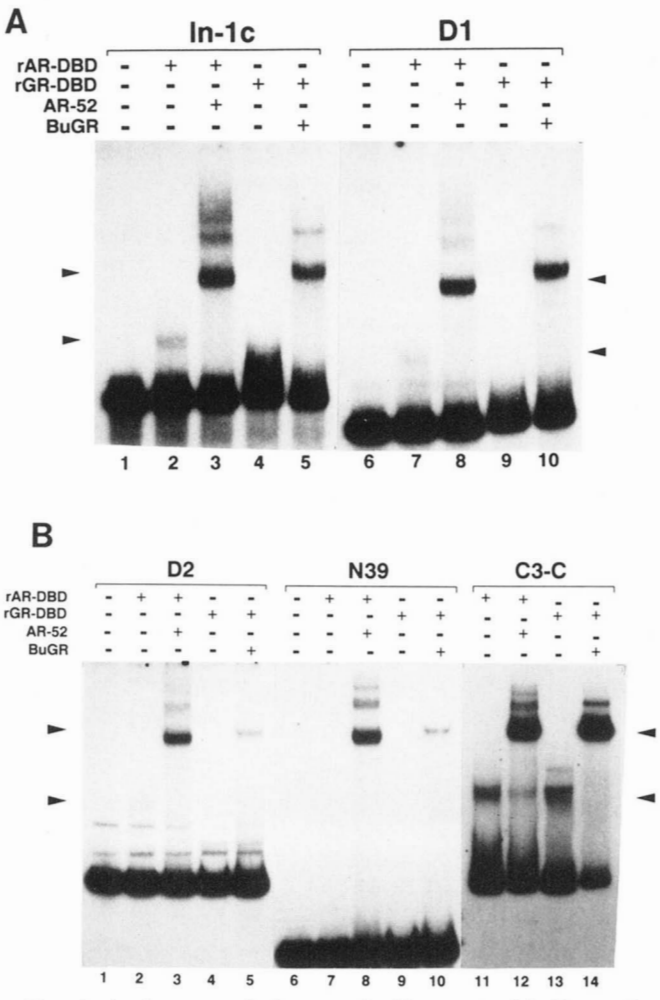

FIG. 8. **Androgen and glucocorticoid receptor binding to In-1c and its sequences D1, D2, and N39.** Each binding reaction contained ^{32}P-labeled probe (10,000 cpm) and 0.5 μg of poly(dI-dC) in a 20-μl total volume. Recombinant rAR-DBD or rGR-DBD partially purified from *E. coli* was incubated with labeled probe and loaded onto 4% (*panel A*) or 5% (*panel B*) nondenaturing polyacrylamide gels. *Panel A*: *lanes 1–5* contained ^{32}P-labeled In-1c; *lanes 6–10* contained ^{32}P-labeled D1; *lanes 1* and *6*, free labeled probe; *lanes 2*, *3*, *7*, and *8*, 1.0 μg of total protein of rAR-DBD; *lanes 4*, *5*, *9*, and *10*, 2.0 μg of total protein of rGR-DBD; *lanes 3* and *8*, 1.2 μg of protein of antibody AR-52; *lanes 5* and *10*, 2 μl of a 1:5 dilution of BuGR antiserum. *Panel B*: *lanes 1–5* contained ^{32}P-labeled D2; *lanes 6–10* contained ^{32}P-labeled N39; *lanes 11–14* contained ^{32}P-labeled C3-C; *lanes 1* and *6*, free labeled probe; *lanes 2*, *3*, *7*, *8*, *11*, and *12*, 1.0 μg of total protein of rAR-DBD; *lanes 4*, *5*, *9*, *10*, *13*, and *14*, 2.0 μg of total protein of rGR-DBD; *lanes 3*, *8*, and *12*, 1.2 μg of protein of antibody AR-52; *lanes 5*, *10*, and *14*, 2 μl of a 1:5 dilution of BuGR antiserum. Note that for assays with In-1c and its subfragments, rAR-DBD and rGR-DBD and their corresponding antibodies were used in the same amounts in *A* and *B* as those used for C3-C.

complex androgen response element. Intron 1 fragment In-1c was a stronger androgen-dependent enhancer in CV1 cells than two other first intron regions, In-1a and In-1b, each selected by the presence of GRE-like sequences. GRE-like sequences were noted also in the 5′-flanking region; however, this region was less responsive than either the large first intron fragment In-1 or its subfragment In-1c. Low levels of androgen-stimulated CAT activity were observed with two reporter constructs each containing fragments of the 5′-flanking region in ptkCAT. However, the 5′-flanking DNA (−901 to +8) with its own promoter (cloned in pCAT) was not an effective androgen-dependent enhancer of transcription. We observed a similar lack of androgen regulation of the C3 subunit gene promoter in CV1 cells (10).

Several features of the first intron element In-1c are noteworthy. Although In-1c bound both AR and GR in the DNA mobility shift assay, only AR induced a transcriptional response in CV1 cells. A cluster of GRE-like sequences, N39, consisting of a 15-bp partial palindrome and two right half-sites, when isolated from In-1c functioned as a weak ARE or GRE, although it bound AR better than GR. Thus the selective ARE activity of In-1c was likely determined by AR interactions with other nucleotide sequences or with one or more nonreceptor regulatory proteins interacting outside the GRE-like cluster. In CV1 cells, ARE specificity of the D1 and D2 subfragments of In-1c was retained even though they also bound AR and GR. However, transfection of reporter vectors containing In-1c, D1, or D2 into PC3 and HeLa cells revealed differences in their transcriptional responses. In PC3 and HeLa cells, In-1c and D2 were less responsive to AR than in CV1 cells. In-1c also mediated a weak response to GR in these cells, whereas D2 remained AR-specific. D1 was a weaker ARE than In-1c or D2 in all three cell lines. It exhibited GRE activity only in PC3 cells and at a lower level than ARE activity. The selective AR responsiveness of D2 in the three different cell lines suggests that it is intrinsically AR-specific and is consistent with its stronger binding of AR than GR. However, the AR specificity of In-1c was confined to CV1 cells. Thus, the In-1c subfragments, D1 and D2, combined to make In-1c a stronger ARE than GRE in PC3 and HeLa cells. The relatively weak GRE activity of In-1c could result from a permissive factor for GR transactivation which is not present in CV1 cells (34). The factor might be a specific GR coactivator or an inhibitor of a selective GR suppressor.

The absence of In-1c GRE activity in CV1 cells and its presence in HeLa cells are reminiscent of studies on a composite GRE of the proliferin gene that contains an AP1 recognition sequence and responded with repression or stimulation of reporter gene expression depending on the relative amounts of c-*Jun* and c-*Fos* in the cell (16, 35–39). In CV1 cells, repression of GR transactivation was linked to the predominance of *Fos/Jun* dimers, whereas in the HeLa cells, GR-dependent enhancement of reporter gene expression was related to the presence of AP1 as *Jun/Jun* homodimers (16). In-1c mimics this pattern of cellular response in that it is a GRE in HeLa cells but not in CV1 cells. However, subfragment D2, which contains both AP1 and CREB recognition sites, did not mediate a response to GR in either HeLa or CV1 cells, suggesting that factors other than or in addition to *Jun* and *Fos* may influence the receptor specificity of this complex element.

AR binding to In-1c and its subfragments D1 and D2 was weaker than to the 15-bp partial palindrome ARE within intron 1 of the C3 subunit gene (10). Low receptor binding activity is a feature of the complex ARE controlling the mouse sex-limited protein gene (17) and of complex response elements described for GR (15, 16). GRE-like sequences within In-1c, although not tested individually, would be considered weak GREs according to the nucleotide sequence criteria of Nordeen *et al.* (40). In general, it has been found that within this family of simple response elements the 15-bp partial palindromes have both ARE and GRE activities (7, 14). Based on a consensus sequence (10, 14, 40), the strongest simple elements of In-1c are in the 39-bp sequence at the 5′ end of the D1 subfragment, and they too were relatively weak as an isolated unit when analyzed in CAT assays. The absence of strong individual response elements within a complex element may allow for modulation by different factor interactions (17),

thus enabling the element In-1c to mediate AR-specific transactivation in CV1 cells and yet transact responses to both AR and GR in PC3 and HeLa cells. Interestingly, D2 was a stronger ARE than D1 yet lacks a sequence conforming to that of a consensus ARE/GRE (7, 12, 40). The closest to an ARE/GRE-like 15-bp partial palindrome is GTCATACTTTGTAAC (nucleotides 2614–2600, 5′ to 3′ on the antisense strand). It remains to be determined if this is an effective AR binding site.

Winderickx *et al.* (23) demonstrated that androgen regulation of 20-kDa protein gene expression requires protein synthesis. The testosterone-stimulated increase in 20-kDa protein mRNA was blocked by cycloheximide, indicating that rapidly turning over factors might interact with AR and GR to control transactivation. The multiplicity of potential controlling elements in In-1c provides a structural framework for cooperativity among ARE/GRE-like sequences as well as interactions with transcription control sequences (Tables I and II). Functional cooperativity was demonstrated among GREs and recognition sequences for several transcription-regulating proteins including NF1, SP1, CCAAT box, OTF, and CACCC box-binding proteins (41–43). NF1 appears to have no direct interaction with GR; however, the function of OCT-1, another transcription factor that activates the mouse mammary tumor virus promoter, requires direct interaction with the receptor (44). In-1c contains an OCT-1 sequence as well as potential recognition sequences for the known factors C/EBP, AP1, CREB, and AP2. C/EBP elements are located within a glucocorticoid regulatory unit of the rat α-acid glycoprotein gene (21). AP1 sites belong to an overlapping set of response elements for *Jun/Fos*, CREB, and might also mediate effects of cyclic AMP. Rat ventral prostate contains a high density of β-adrenergic receptors, and adrenergic stimulation of adenylate cyclase and prostatic binding protein gene expression has been demonstrated (45, 46). In the glutamine synthetase gene, glucocorticoid induction of transcription required coordination of a GRE with an AP1/ATF-CRE-like element (47).

Within In-1c there are a number of inverted and direct repeats that may not only serve as recognition sites for cell-specific factors but also provide a secondary structure that influences receptor interactions with other transcription control proteins. It is not uncommon for inverted and direct repeats to be associated with hormone response elements or other transcription control elements (10, 19, 48). In the D2 subfragment of In-1c, for example, a 7-nucleotide AT-rich inverted repeat (Fig. 6) encompasses the only candidate ARE in this subfragment. Similarly in D1 there is a GC-rich 10-nucleotide inverted repeat that could, by looping out, bring several weak AREs into closer proximity. D1 also contains an interesting alternating purine-pyrimidine sequence with flanking dyad symmetry (Fig. 6).

The presence of both AR and GR in rat ventral prostate epithelial cells provides a capability for overlapping effects of androgen- and glucocorticoid-induced gene regulation. Glucocorticoids in ventral prostate cells act through GR since their binding to AR is negligible (49). Both glucocorticoids and androgens stimulate prostatein secretion and plasminogen activator activity in short term cultures of ventral prostate (50–51). However, treatment of castrate rats with hydrocortisone only partially replaces the effects of androgen in preventing prostate weight loss and the decrease in prostatein C1 subunit mRNA as well as increases in plasminogen activator activity and mRNAs for TRPM-2 and c-*fos* (52). GR may have an important role in modulating the expression of androgen-regulated genes in prostate and other cells in which AR and GR coexist. If so, the effect of GR competition with AR for a common response element would depend on the nature of the element. Simple elements like the first intron element of the C3 subunit gene (10) could mediate equivalent positive responses to AR and GR. However, with complex elements such as In-1c and others (12, 18) which bind GR and AR but respond preferentially to AR, GR could inhibit AR transactivation. Development and maintenance of prostate functions *in vivo* are androgen-dependent, and the prostate gland undergoes involution following androgen withdrawal despite continuing secretion of adrenal corticosteroids. Thus, in the absence of androgen *in vivo*, positive transactivation functions of GR which are dependent on the presence of androgen-regulated transcription factors would become ineffective. Recent studies have indicated the involvement of androgen-induced DNA-binding proteins in androgen control of the mouse kidney β-glucuronidase (19) and RP2 genes (53). Androgen-regulated transcription control factors are likely to be the subject of intensive investigation in future years.

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